Enhanced Revascularization of the Ischemic Limb by Angiogenic Therapy

Li-Qun Pu, MD; Allan D. Sniderman, MD, FRCP(C); Roland Brassard, MD, FRCP(C); Kevin J. Lachapelle, MD; Alan M. Graham, MD, FRCS(C); Robert Lisbona, MD, FRCP(C); and James F. Symes, MD, FRCS(C)

Background. This study tests the efficacy of an angiogenic growth factor, endothelial cell growth factor, in a rabbit model of persistent hindlimb ischemia.

Methods and Results. Ischemia was induced in the left hindlimb of 22 New Zealand White rabbits by ligation of the distal external iliac artery and complete excision of the common and superficial femoral arteries. Two groups of animals were studied: Group 1 consisted of 11 animals who for 10 days received daily intramuscular injections of 4 mg of endothelial cell growth factor beginning on postoperative day 11, and group 2 consisted of 11 animals who underwent the same surgical ischemic procedure but received only injections of saline daily for the same postoperative period. Perfusion of the ischemic left limb was compared with the normal right limb in each animal on postoperative days 10, 20, 30, and 40 using the calf blood pressure ratio, $^{99m}$Tc macroaggregate radioisotopic perfusion scans, and serial angiography. Neovascularization in the left thigh at day 40 was quantified from the angiograms. Each technique documented that animals in group 1 had significantly better perfusion than animals in group 2; that is, the calf blood pressure ratio was higher in group 1 than in group 2 (0.56 versus 0.32 at day 20, 0.64 versus 0.44 at day 30, and 0.70 versus 0.50, $P<.0001$), and the calf radioisotopic perfusion ratio was also higher in group 1 than in group 2 (0.88 versus 0.74 at day 20, $P<.02$; 0.93 versus 0.76 at day 30, and 0.96 versus 0.79 at day 40, $P<.008$). Angiographic studies correlated well with these results demonstrating much earlier distal arterial reconstitution and enhanced neovascularization (23.8 versus 9.0 vessels, $P<.007$).

Conclusions. The data clearly indicate that an angiogenic growth factor, endothelial cell growth factor, promotes revascularization in this experimental ischemic hindlimb model, raising the possibility that in the future such agents might be of value in humans. (Circulation 1993;88:208-215)

Key Words • angiogenesis • ischemia • collaterals • growth factor • revascularization

The number of people who suffer from lower-extremity ischemia is increasing with a corresponding increase in the need for limb salvage vascular surgery.1 Currently, severe chronic limb ischemia is treated by a variety of arterial revascularization techniques, including bypass procedures using autogenous and synthetic conduits, endarterectomy, and percutaneous techniques such as balloon angioplasty and atherectomy. Unfortunately, about 20% of patients with limb-threatening ischemia have disease that is so extensive that direct revascularization procedures cannot be undertaken successfully.2 Too often, the only alternative for these patients is limb amputation with its attendant morbidity and mortality.

A number of innovative approaches have been reported clinically and experimentally. These include pedal vessel bypass,3 conventional bypass with adjunctive distal arteriovenous fistula,4,5 staged arteriovenous reversal,6-9 omental or muscle flap transfer,10-12 and others.13 However, the effectiveness and practicality of all such procedures have yet to be clearly established. Powerful testament to the size of the problem that remains comes from the Maryland study by Tunis et al,14 which showed no decrease in the amputation rate despite the increase in bypass surgery and angioplasty.

Angiogenesis is the term that describes the formation of new blood vessels. Folkman15,16 and others17 have not only elucidated some of the fundamental biologic mechanisms involved in angiogenesis but have also pioneered the isolation and description of a number of angiogenic factors.18,19 Recently, the studies by Thompson et al,20,21 Goldsmith et al,22,23 and others24-27 in different animal models demonstrated dramatic angiogenesis in response to the administration of one or other of the angiogenic factors. These results stimulated our interest in the possibility of using such an approach in the treatment of severe chronic limb ischemia. The purpose of the present study was to test the hypothesis that administration of one of these factors, endothelial cell

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From the Cardiovascular Research Laboratory, Departments of Surgery (L.-Q.P., K.J.L., A.M.G., J.F.S.), Medicine (A.D.S.), Radiology (R.B.), and Nuclear Medicine (R.L.) Royal Victoria Hospital and McGill University, Montreal, Quebec, Canada.

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Address for reprints: James F. Symes, MD, Department of Surgery, St. Elizabeth’s Hospital of Boston, 736 Cambridge St, Boston, MA 02135.
growth factor, would significantly improve perfusion in a rabbit ischemic hindlimb model.

Methods

Animal Hindlimb Ischemic Model

Twenty-two adult New Zealand White male rabbits (mean weight, 4 kg) were studied. All animals were anesthetized with ketamine (50 mg/kg i.m.) and xylazine (5 mg/kg i.m.), and a longitudinal incision was made in the left groin from the inguinal ligament to above the knee. With the aid of surgical loops, the femoral artery was dissected, and its branches, the profunda, the lateral circumflex, and the superficial epigastric were exposed as completely as possible. The proximal popliteal and saphenous arteries also were dissected free. Ischemia then was induced in the left hindlimb by ligation of the distal external iliac artery just above the inguinal ligament, the inferior epigastric artery, all the branches of the femoral artery, and the proximal popliteal and saphenous arteries. This was followed by excision of the common and superficial femoral arteries (Fig 1). All animals received 50 mL of 0.9% sodium chloride during surgery and cefazolin (15 mg·kg⁻¹·d⁻¹ i.m.) for 4 days starting on the day of the surgery. An analgesic, buprenorphine (0.04 mg/kg), was administered daily for the first 10 days after surgery. Approval for animal use in this study was granted by the institutional animal care committee, and the care of these animals complied with the guidelines of the Canadian Council of Animal Care, the “Principles of Laboratory Animal Care,” and the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 80-23, revised 1985).

Endothelial Cell Growth Factor

Endothelial cell growth factor is purified from bovine retina and is one of the heparin-binding growth factors.¹⁸,¹⁹ Endothelial cell growth factor is an acidic fibroblast growth factor that has been shown to promote endothelial cell proliferation in vitro (at a concentration of 1 ng/mL) and to stimulate angiogenesis in vivo (at levels of 10 to 100 ng in the chick chorioallantoic membrane and corneal bioassays).¹⁸,²⁰

Experimental Protocol

Two groups of 11 rabbits each were studied. Group 1 received 4 mg of endothelial cell growth factor (EndoGro™ VECTEC Inc, Albany, NY; 100 mg + 10 units heparin per vial) in 3 mL saline i.m. in the left thigh daily for 10 days commencing on postoperative day 11. Group 2 animals received a sham injection of 3 mL saline i.m. according to the same schedule. The animal was evaluated preoperatively (day 0) and on postoperative days 10, 20, 30, and 40 by clinical assessment, calf blood pressure, calf radioisotopic perfusion, and angiography. The study was terminated on postoperative day 40.

Study Parameters

Calf blood pressure. Calf blood pressure was measured in both limbs by Doppler flowmeter (model 1059, Parks Medical Electronics, Aloha, Ore). The hindlimbs were shaved and cleaned, and under anesthesia as described above, the pulse of the posterial tibial artery in the lower calf was detected by the Doppler probe; systolic blood pressure then was determined. The calf blood pressure is defined as the ratio of the left calf to right calf systolic pressure (L/R ratio).

Calf radioisotopic perfusion scan. Arterial perfusion was determined radioisotopically using ⁹⁹mTc macroaggregates that measured 15 to 30 μm in diameter (E.I. du Pont de Nemours & Co., Boston, Mass). The aggregates are designed to be so large that they will be trapped in the capillary bed. With the animal under anesthesia, the left ventricle was punctured through the fourth intercostal space, and 1 mCi of ⁹⁹mTc macroaggregates in 2 mL saline was injected. After the injection, the limbs of the animal were scanned and counted on a gamma camera (Omega 500, Technicare Co., Cleveland, Ohio) that was interfaced to an MCS 560 computer system. The accumulated counts were stored on a disk for later retrieval and analysis. At that time, the radioactive counts from each calf were generated from corresponding regions of interest, and the ratio of the counts between the calves (L/R ratio) was calculated as an index of relative calf perfusion.

Angiography. Angiograms were performed using standard techniques. Two animals were studied for each group at postoperative day 20, and four from each group were studied at day 40. With the animals under anesthesia, the left common carotid artery was exposed through a ventral incision in the neck. By the Seldinger

<table>
<thead>
<tr>
<th>Table 1. Calf Blood Pressure (Left-to-Right) Ratio</th>
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<tr>
<td>Day</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>Group 1</td>
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<td>Group 2</td>
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Values are given as mean±SEM.

*P<.0001 day 10 versus day 0.
†P<.0001 group 1 versus group 2.
TABLE 2. Calf Blood Flow (Left-to-Right) Ratio With Radiolabeled Microaggregate Perfusion Scan

<table>
<thead>
<tr>
<th>Day</th>
<th>Group 1</th>
<th>Group 2</th>
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<tbody>
<tr>
<td>0</td>
<td>0.99±0.02</td>
<td>1.01±0.02</td>
</tr>
<tr>
<td>20</td>
<td>0.88±0.03*</td>
<td>0.74±0.05</td>
</tr>
<tr>
<td>30</td>
<td>0.93±0.04†</td>
<td>0.76±0.05</td>
</tr>
<tr>
<td>40</td>
<td>0.96±0.02†</td>
<td>0.79±0.05</td>
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Values are given as mean±SEM.
*P<.02, †P<.008 group 1 versus group 2.

technique, a 4F catheter was introduced into the exposed artery and advanced to a position 3 cm proximal to the aortic bifurcation. Ten milliliters of contrast agent (MD-76, diatrizoate meglumine) was injected at a rate of 3 mL/s, and serial filming of both hindlimbs was performed.

Vascularization in the left thigh was determined from the angiograms at day 40 and defined as the number of collateral vessels extending along a vertically drawn line that passed through the center of the femur. The 4-second postinjection film was analyzed on two separate occasions by the same observer, and the results were averaged. If the two differed by more than 10%, a third count was made, and the three results were averaged.

Statistical Analysis

All data are expressed as the mean±SEM. Comparisons between group 1 and 2 were performed with a computer statistical package using unpaired Student’s t test. A difference was considered statistically significant if the P<.05.

Results

All animals demonstrated weakness of the left limb on the first postoperative day. After day 10, four of the animals in group 2 developed varying degrees of superficial tissue necrosis in their distal calves or toes. No similar abnormalities appeared in any animals in group 1.

Comparison of calf blood pressure ratios (L/R ratio) determined by Doppler demonstrated no difference between the two groups on postoperative day 0 or 10. In both, severe ischemia was evident on postoperative day 1 or 2. In the left hindlimb compared with preoperative day 0 (P<.0001). However, as shown in Table 1, on all subsequent examinations, although the ratio rose progressively in both groups, the value in group 1 always was substantially higher than in group 2, with the differences easily statistically significant (P<.0001).

The results of the radionuclide perfusion study are given in Table 2. As anticipated, there was no preoperative difference in perfusion between the limbs or between the groups. At the time of the first postoperative examination (day 20), evidence of significantly better perfusion in group 1 than in group 2 was present (0.88 versus 0.74, P<.02). This difference was even

![Fig 2. Angiograms at postoperative day 20 from group 1 (just after endothelial cell growth factor administrations, the fourth second film). Panel A: The early distal arterial reconstitution of the left hindlimb is shown, which accompanies the prominent neovascularization in the left thigh. Panel B: The prominent collateral vessel formation is clearly evident in a magnified picture of the left thigh from the same film.](image-url)
greater on the succeeding examinations such that by day 40, the ratio of flow between the operated and nonoperated limbs in the treatment group approached unity (0.96 versus 0.79, P < .008).

Satisfactory angiographic examinations were obtained in all animals. The examples that follow are representative of the differences that occurred between group 1 and group 2 animals. Figs 2A and 3A were taken on postoperative day 20. Fig 2A is from an animal in group 1, and Fig 3A is from an animal in group 2. Distal arterial reconstitution in the left limb is obvious in the group 1 animal but barely evident in the corresponding angiogram in the group 2 animal. These differences are, if anything, even more obvious in the angiograms focusing on the left thigh. Revascularization is easily evident in group 1 animals (Fig 2B) but virtually absent in those in group 2 (Fig 3B).

The angiograms performed at the end of the study (day 40) also are of considerable interest, again demonstrating substantially more distal arterial reconstitution of the left hindlimb in group 1 animals (Fig 4A) than in group 2 animals (Fig 5A). In addition, more collateral vessels were present in group 1 than in group 2 animals (Figs 4B and 5B, respectively). Of considerable interest as shown in Figs 6A and 6B, a few of the most rapidly "growing" collateral vessels were shown to extend all the way from their original source near the ankle, where they reconstituted the distal arterial tree in that animal.

Quantitative analysis of new vessel formation in the left thigh at day 40 demonstrated more than twice the number of nutrient vessels in group 1 than in group 2 animals (23.8 ± 3.4 versus 9.0 ± 1.5, P < .007).

Discussion

Biotechnology makes it possible to isolate and purify factors that can stimulate new blood vessel growth or angiogenesis. In the present study, several techniques were used to examine and quantify limb perfusion. The results were consistent throughout; in every instance, substantially improved perfusion and revascularization were seen in the group that received the angiogenic factor during the postoperative period.

The agent used in the present study is one of the heparin-binding growth factors. This group has been divided into a basic fibroblast growth factor and an acidic fibroblast growth factor. The genes and protein structure of the two forms are similar, and they bind to the same receptor. In most systems examined, basic fibroblast growth factor is from 10- to 100-fold more potent than acidic fibroblast growth factor. Although heparin has been shown to promote angiogenesis in a large-animal model following continuous intravascular infusion, the apparent dose required far exceeded (2 × 10^4 units per animal) that used in this study (0.4 units per injection). It is highly unlikely, therefore, that the angiogenic effect we observed was related to the small amount of heparin that was bound to the endo-
The endothelial cell growth factor. On the other hand, in the presence of heparin, the ability of acidic fibroblast growth factor to stimulate endothelial cell proliferation increases 100-fold, and under these circumstances, the ED$_{50}$ of acidic fibroblast growth factor is just about the same as basic fibroblast growth factor. In the present study, we used endothelial cell growth factor, which is an acidic fibroblast growth factor. We did so because it is cheaper than basic fibroblast growth factor and is available commercially bound to heparin, so we believed it was likely to be efficacious. Larger doses also could be administered. The doses of the endothelial cell growth factor used in this study were based on previous experience obtained during in vivo studies by Thompson et al. and Andrade et al. The format and schedule of administration of the agent were based on Goldsmith's lipid angiogenic factor study as well as preliminary experience from our laboratory. Administration of the agent was delayed to postoperative day 11 to minimize confounding effects of the host response to acute ischemic injury and the surgical procedure.

Angiogenesis is a complex process involving capillaries and venules, and the exact mechanism by which it was achieved in this study remains unknown. At least four steps have been demonstrated to be involved in the development of a new capillary: enzymatic degradation of the basement membrane of the parent vessel to allow formation of a capillary sprout, migration of endothelial cells toward the angiogenic stimulus, proliferation of endothelial cells just behind the leading front of migrating cells, and, finally, maturation and organization of endothelial cells into capillary tubes. Endothelial cell growth factor might have acted through either one or several pathways to promote this process. For example, as suggested by Folkman and Klagsbrun, it might stimulate and mobilize macrophages that then secrete angiogenic growth factors or chemotactic agents for vascular endothelial cells. Another possibility is that endothelial cell growth factor causes the release of such factors from intracellular sites in cells within the ischemic tissue itself. Alternatively, of course, it might act directly on the capillaries and arterioles of the ischemic vessels to promote the growth and development of new vascular channels.

In this study, we obtained quantitative angiographic evidence of revascularization in the ischemic hindlimb of group 1 compared with group 2 on angiography. The accurate quantitation of revascularization in an ischemic hindlimb model is difficult, but we believe that the method we used in the present study is simpler and more reliable than other techniques that are used to quantitatively investigate in vivo angiogenesis in an ischemic limb model of large animals. However, the use of the available techniques to determine whether the increased collateral vessels seen on angiograms are

Figure 4. Angiograms at postoperative day 40 from group 1 (20 days after endothelial cell growth factor administrations, the fourth second film). Panel A: The more distal arterial reconstitution in the left hindlimb is evident, which accompanies the more visualized neovascularization in the left thigh compared with group 2. Panel B: The significantly increased collateral vessel formation is demonstrated in a magnified picture of the left thigh from the same film.
FIG 5. Angiograms at postoperative day 40 from group 2 (the fourth second film). Panel A: Poorly developed collateral vessels (arrows indicate the sources of these collaterals) and distal arterial reconstitution in the left hindlimb are evident compared with group 1. Panel B: A magnified picture of the left thigh from the same film.

newly formed or just enlarged pre-existing vessels still presents a challenge.

The cellular events in the new vessel formation process currently are thought to be under the control of locally acting growth factors. The study by Mooney et al suggested that local administration of an angiogenic factor (tumor necrosis factor) demonstrated beneficial effects superior to its systemic application in a rat wound-healing model. The topical application of angiogenic factors has been used by several investigators to enhance wound healing, bone graft healing, vascular graft endothelialization, bronchial anastomotic healing after lung transplantation, duodenal ulcer healing, and peripheral nerve regeneration. Whether any of the angiogenic factors used in these local applications might also have a systemic effect remains uncertain.

Regardless of the mechanism, the results of the present study strongly suggest that agents such as endothelial cell growth factor have the potential to markedly enhance angiogenesis in the presence of limb ischemia. Each technique used to assess this phenomenon confirmed the efficacy of this agent in this experimental model of persistent limb ischemia. The angiographic studies in particular provide graphic evidence of enhanced neovessel formation in the group receiving endothelial cell growth factor. Curiously, until quite recently, this class of agents had not been tested experimentally for its capacity to relieve organ ischemia. Some evidence is now available, however, with regard to both the heart and hindlimbs. Banai et al, for example, were unable to demonstrate benefit from delivery of an acidic fibroblast growth factor to ischemic myocardium from an epicardial sponge when regional ischemia was produced gradually by application of an anaeroid constrictor to the left anterior descending coronary artery. By contrast, Yanagisawa-Miwa et al demonstrated significantly enhanced collateralization with intracoronary injection of a basic fibroblast growth factor. The first report, however, of successful revascularization of the ischemic hindlimb due to administration of an angiogenic factor came from our laboratory, an observation that since has been confirmed by Baffour and colleagues using, however, a basic fibroblast growth factor.

Clearly much remains to be done to further clarify and confirm the efficacy of this approach to limb as well as other tissue revascularization. In addition, the safety and potential adverse effects of administration of such agents must be assessed because the animals in the present study were not systematically assessed for evidence of hematological, renal, or hepatic toxicity after administration of endothelial cell growth factor. Nevertheless, given this present evidence of in vivo efficacy and the obvious need to improve our therapy of severe arterial insufficiency in patients, clinical application in the future of angiogenic agents similar to this should be considered a real possibility.
FIG 214 Circulation ment; 5.4.2.7.6.7. The authors of the article would like to thank Dr. Phina Brodt, Department of Surgery, and Dr. Bernard I. Weigensberg, Department of Pathology, for their help and discussion in this work; Ms. Lina Pepe for her assistance in performing the experiment; and Mrs. Lucie Francoeur for preparing the manuscript. Dr. Puj was a recipient of a Renouf Fellowship from the Research Institute of Royal Victoria Hospital, McGill University, during the period of the study.

References

Fig 6. An angiogram at postoperative day 40 from a group 1 animal. Panel A: The rapid “growing” collateral vessels (arrows) from their original branches in the left thigh are clearly demonstrated in the distal second film. Panel B: A number of the far distal collateral vessels (arrows) are evident toward the ankle to reconstitute the distal arterial trees in a magnified picture of the left calf in the fifth second film.


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